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Comparison of soy lecithin, coconut water, and coconut milk as substitutes for egg-yolk in semen cryodiluent for black rhinoceros (*Diceros bicornis*) and Indian rhinoceros (*Rhinoceros unicornis*)

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ABSTRACT

Semen cryopreservation for the black rhinoceros (*Diceros bicornis*) and Indian rhinoceros (*Rhinoceros unicornis*) relies on extenders containing egg-yolk (EY). Use of such media is not ideal as inter-batch composition varies and there is risk of pathogenic contamination. The goal of this study was to test animal protein-free extenders. Semen collected via electroejaculation from 10 rhinoceros (6 black, 4 Indian) was diluted with extender containing EY, 1% or 2% soy lecithin (1%SL; 2%SL), coconut water (CW), or coconut milk (CM), cryopreserved and evaluated for sperm motility, viability, morphology, progression, and acrosomal integrity at 0, 1, 3, 6 and 24 h post-thaw. Mean ± SD fresh ejaculate motility was 84.5 ± 7.6%, progression: 3.6 ± 0.6 (scale 0–5), viability: 83.4 ± 7.1%, intact acrosomes: 71.3 ± 6.9%, and morphologically normal: 78.8 ± 13.6%. Motility and progression decreased in all groups post-thaw, were greatest in EY, and decreased over time ($P \leq 0.05$). Motility and progression did not differ ($P > 0.05$) between 1%SL and 2%SL, but were lower ($P \leq 0.05$) in CM and CW, and acrosomal integrity was higher ($P \leq 0.05$) in EY, 1%SL and 2%SL than in CM and CW. Post-thaw viability was greatest in EY and 2%SL followed by 1%SL, then CM and CW ($P \leq 0.05$). Morphology did not differ among treatments ($P > 0.05$). Morphology, acrosomal integrity, and viability were maintained over time ($P > 0.05$). Although some rhinoceros sperm survived cryopreservation in SL treatments, reduced post-thaw motility rendered all treatments inadequate substitutes for EY-based extenders.

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1. Introduction

The black rhinoceros (*Diceros bicornis*) and Indian rhinoceros (*Rhinoceros unicornis*) are considered critically endangered and vulnerable, respectively, by the IUCN [1,2]. Successful captive breeding programs may soon play an important role in promoting species survival, as poaching and habitat loss, among other threats, severely hinder population growth *in situ*. Semen cryopreservation in tandem with other assisted reproductive technologies (ARTs) can facilitate genetic management [3,4] and though semen collection and cryopreservation protocols have been developed for black, white (*Ceratotherium simum*), Indian, and Sumatran (*Dicerorhinus sumatrensis*) rhinoceros, they rely on extenders that contain an animal protein, egg-yolk (EY) [5–10].

The use of animal protein, such as EY or skim milk, in semen extenders is common practice [11]. EY protects spermatozoa from damage during cryopreservation, as is often evidenced by increased viability, motility and fertilizing capacity post-thaw when compared to extenders that do not contain EY [11,12]. Membrane composition, particularly cholesterol and phospholipid content, and the interaction of various cryoprotectant agents and extenders with the membrane components, greatly influence cryosurvival [12–16]. It is generally accepted that the protective effect provided by EY is attributable to low-density lipoproteins (LDLs) and phospholipids [11,17], though direct mechanism is unclear. There is speculation that EY may stabilize the membrane, replace phospholipids lost during the cryopreservation process [15], or prevent the efflux of cholesterol and phospholipids [11].

The efficacy of animal protein based media has come into question prompting the search for an equally effective vegan or chemically defined alternative [11,12,18–21]. EY composition can vary among batches, potentially influenced by the diet and health status of the hen [22], whereas plant composition can be better controlled. More importantly, there is risk of bacterial or viral

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contamination in EY, which may be a source of endotoxins potentially impacting fertilizing capacity of the spermatozoa [23] and the transport of samples containing animal protein could result in the spread of disease [24].

Soy lecithin (SL) plays a role equivalent to EY, protecting the sperm membrane during cryopreservation [12] by possibly mitigating the efflux of cholesterol or phospholipids. Phosphatidylcholine, a key component of SL, performed equally well to EY as an additive to extenders for stallions [25] and it is hypothesized that any extenders with choline phospholipids will improve spermatozoa cryosurvival [11]. SL is an effective alternative to EY in semen cryopreservation for many species including humans [26], bulls [18], stallions [27], goats [28], rams [29], and domestic cats [21] and dogs [30]. SL concentrations appear optimal at approximately 1% of extender volume [21,26,28–30], resulting in high sperm quality post-thaw; higher concentrations appear to have a toxic effect [28,29]. No bacterial contamination was found in samples that used SL as a diluent [23]. Furthermore, fertility rates of cows were equivalent when inseminated with semen frozen with SL as compared to an EY based extender [23]. Bull sperm frozen using commercially available SL extender displayed higher motility and equal measures of viability, acrosomal integrity, and acrosome reaction induction as compared to samples frozen using EY based diluents [18]. However, contrasting reports indicate the use of SL extenders resulted in decreased motility in bulls [31] and mitochondrial damage in rams [19].

Coconut (*Cocos nucifera*) water (CW), the liquid endosperm of green coconuts, and coconut milk (CM), the extract of fruit pulp from mature coconuts, contain many fatty acids (including poly unsaturated fatty acids (PUFAs)), amino acids, antioxidants, vitamins, minerals, and sugars, contributing to their usefulness as semen extenders and culture media [32–34]. PUFAs are thought to contribute to fluidity and flexibility of membranes and, as precursors to prostaglandins and leukotrienes, may influence motility [13,16]. Incorporating PUFA, docosahexaenoic acid (DHA) to extenders, increased membrane integrity and sperm motility in boars [35]. Furthermore, CW is an effective diluent for cryopreservation of ram [36] and goat [37] semen. CM prolonged bull sperm viability at room temperature when used as an additive to a citrate buffer medium [38], and did not impact fertilizing capacity [39]. When used in addition to EY, CM did not impact fertility rates following AI in goats [40], protected against acrosome damage in boars [41], and promoted bull sperm viability over time in chilled samples [42].

The goal of this study was to evaluate soy lecithin, coconut water, and coconut milk as animal protein-free alternatives to egg-yolk in traditional rhinoceros semen extenders. Sperm quality measures including motility, progressive status, viability, acrosomal integrity, and morphology, were used to assess the efficacy of the substitutes following cryopreservation of black rhinoceros and Indian rhinoceros spermatozoa.

2. Materials and methods

2.1. Animals and semen collection

All procedures were reviewed and approved by the Cincinnati Zoo and Botanical Garden's Animal Care and Use Committee (protocol #14-120). Semen was collected via electroejaculation [8,10] from 10 adult male rhinoceros (6 black, 4 Indian; 1 ejaculate/male) maintained at institutions across the United States (Table 1). A surgical plane of anesthesia was induced in each male, though specific drug combinations varied (Table 1). Rectal probes designed specifically for each rhinoceros species [8] (Innovative Zoological Solutions, Cincinnati, OH 45205, USA) with 3 longitudinal electrodes and an electroejaculator (Innovative Zoological Solutions; P-T Electronics, Boring, OR 97009, USA) were used to provide stimuli over the course of 2–3 series (Table 2). Each series was followed by a resting period of at least 5 min, during which motility and progressive status of each sample were assessed. Semen was collected into whirlpak bags (Nasco, Fort Atkinson, WI 53538, USA) that were frequently changed between stimulations and stored in an insulated container until prepared for cryopreservation. Prior to cryopreservation, samples were assessed for motility and progressive status, and sperm concentration was determined using a hemocytometer (American Optical, Buffalo, NY 14215) [43].

2.2. Semen cryopreservation

Unless otherwise indicated, all chemicals were obtained from Sigma Aldrich (St. Louis, MO 63146). Semen samples (exhibiting $\geq$ 60% motility) were prepared for cryopreservation as described in O'Brien and Roth [6]. Briefly, 250 μL of semen per treatment group were diluted 1:1 with semen extender and contained in a water bath at room temperature (RT). Treatment groups differed by extender type and included a control which employed an equine extender (EQ) containing EY (20% v/v), lactose (5.5% v/v), disodium EDTA (0.25% w/v), glucose (1.5% w/v), Equex STM (0.25% v/v; Nova Chemical, Moon Township, PA 15108, USA), 25 iu penicillin G mL^{-1}, and 25 iu streptomycin mL^{-1} [6]. Soy lecithin (1%SL and 2%SL), coconut milk (20% v/v; CM), and coconut water (20% v/v; CW) were used in lieu of EY for the respective treatment groups. The source of SL (Swanson health products, Fargo ND) and concentrations used were chosen based on the beneficial effects reported by similar studies in other species [21,26,28–30]. The CW (Harmless Harvest, San Francisco, CA 94111, USA) and CM (Native Forest, Edwards and Sons, Carpinteria, CA 93014, USA) were organic and did not contain any preservatives or additives. All extenders were brought up to a pH of 7.0 and stored at −80 °C until use. Samples were cooled to 4 °C then diluted further 1:1 with chilled extender containing 10% glycerol in a stepwise manner (25, 25, 50% v/v every 20 min) to a final concentration of 5% glycerol and final volume of 1 mL per treatment. Samples equilibrated for 1 h at 4 °C, and were loaded into cooled 0.5 mL straws (2 straws/treatment/male), placed into cooled canes and lowered into a charged dry shipper (depth: 42 cm, capacity: 3.6 L; Chart MVE Biomedical, Ball Ground, GA 30107) for 10 min [44] before being plunged into liquid nitrogen and stored until post-thaw assessment.

Straws were thawed in random order and treatment group was not revealed to the assessor until after all assessments were complete. All assessments were conducted by the same individual for consistency. Two straws of each treatment group were processed and evaluated separately, and therefore each assessment was carried out in duplicate. Values from duplicate straws were averaged for each individual prior to statistical analysis. Straws were thawed (10 s at RT in air; 20 s in 37 °C water bath) and maintained, protected from light, at RT, then evaluated for sperm motility, viability, morphology, progressive status, and acrosomal integrity at 0, 1, 3, 6 and 24 h post-thaw.

2.3. Motility and progressive status assessment

The percentage of motile sperm was assessed on pre-warmed slides under 200× magnification using phase contrast optics (Axiostar plus; Carl Zeiss Microscopy, Thornwood, NY 10594). Progressive status, straight-forward movement, was ranked on a scale of 0–5 (5 = all sperm exhibiting rapid, forward progression).

2.4. Acrosomal integrity assessment

To assess acrosomal integrity, a 5 μL aliquot of each sample was

Table 1
General characteristics, locations, and immobilization details for electroejaculation semen collection of Indian and black (Southern and Eastern subspecies) rhinoceros.

		SB[a]	Age (Years)	Proven?[b]	Institution	Location	Immobilization Drugs[d]
Indian		327	13.8	Yes	CCTU[c]	Punta Gorda, FL	a, b, e, mt
		330	13.4	Yes	CCTU[c]	Punta Gorda, FL	a, b, e
		364	12.8	Yes	The Wilds	Cumberland, OH	e, d, f, k, md
		414	7.6	No	Buffalo Zoo	Buffalo, NY	e, d, f, k
Black	Southern	670	20.4	Yes	Tanganikya Wildlife Park	Goddard, KS	e, k, md, mt
		846	16.4	No	Chehaw Zoo	Albany GA	e, k, md, mt
		858	16.3	No	Chehaw Zoo	Albany, GA	e, md, mt
		868	14.9	No	Fossil Rim	Glen Rose, TX	e, k, md, mt
	Eastern	870	15.2	No	Sedgwick County Zoo	Wichita, KS	a, e
		946	9.1	No	Caldwell Zoo	Tyler, TX	a, e, k

[a] SB = Studbook.
[b] Proven indicates an individual that has sired a live calf.
[c] CCTU = Center for Conservation of Tropical Ungulates.
[d] azaperone (a), butorphenol (b), detomidine (d), etorphine (e), flunixin (f), ketamine (k), midazolam (md), medetomidine (mt).

smeared onto a slide and allowed to air dry, stored in the dark at 4 °C until processing, and stained with fluorescein-conjugated Arachis hypogeal (peanut) agglutinin (FITC-PNA) [45]. FITC-PNA stain (20 μL; 0.05 μg/mL) was applied to each slide, then slides were incubated in a humidified chamber in the dark at RT for 30–60 min, rinsed with PBS, and allowed to air dry. Once dry, 5 μL of fluoromount (Sigma Aldrich, St. Louis, MI, 63146, USA) was added to each slide and covered with a cover slip. Slides were evaluated using fluorescent microscopy (400×; 100 spermatozoa/straw). Acrosomes were classified as 'intact' if there was staining at the apical ridge, as 'damaged' if staining was partial or mottled and as 'non-intact' if staining only occurred in the equatorial region or was completely missing [10,45].

2.5. Morphological assessment

Morphological assessment was conducted as previously described [6,10,46]; 5 μL of each sample were fixed in 0.3% glutaraldehyde (50 μL). Wetmount slides were examined under 400× magnification using phase contrast optics (100 spermatozoa/straw). Sperm displaying normal morphology were considered 'normal' and those displaying abnormalities such as proximal and distal droplets, coiled tails, micro- or bi-cephaly, etc. were considered 'abnormal'. Abnormalities were divided into primary defects, those impacting the head and midpiece, such as micro-, macro-, and bi-cephaly or missing/damaged midpieces, and secondary defects including proximal and distal droplets, or bent midpieces and tails.

2.6. Viability assessment

The percentage of sperm with intact plasma membranes, indicative of viability, was assessed using 10 μL eosin-nigrosin live/dead exclusion stain (Jorgensen Laboratories, Inc., Loveland, CA 80538, USA) added to 5 μL of sample [47]. Samples were incubated at RT for approximately 10 s before being smeared across a slide and allowed to air dry. Slides were evaluated under 400× bright-field magnification (200 spermatozoa/slide). Sperm displaying no incorporation of stain were considered viable; those displaying partial or full incorporation were deemed non-viable.

2.7. Statistical analysis

Statistical analysis was performed using SPSS for Windows (Version 24; IBM Corp. Armonk, NY). Sperm quality measures (motility, progression, etc.) were analyzed using repeated measures ANOVA followed by post-hoc Bonferroni for pairwise analysis. Mauchly's test indicated that the assumption of sphericity had not been violated, $P > 0.05$, for any of the parameters tested (motility, progressive status, viability, acrosomal integrity, and morphology). The assumption of normal distribution, required for ANOVA, is violated due to small sample size. However, due to lack of a better alternative (parametric or non-parametric) test, repeated measures ANOVA appears to be the most appropriate. Two straws were assessed per treatment per male to account for variability among straws. The mean value of the two straws was used for statistical analysis and therefore, sample size is based upon number of animals. Sperm quality parameters, both fresh and post-thaw, did not differ by species ($P > 0.05$) and species and treatment interaction

Table 2
Fresh ejaculate characteristics and electroejaculation semen collection parameters for black (n = 6) and Indian (n = 4) rhinoceros.

	Black rhinoceros		**Indian rhinoceros**		**Overall**
Characteristics	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD
Seminal volume (mL)	69.5 ± 34.1	43.0–128.8	73.3 ± 61.3	2.8–139.0	71.0 ± 43.6
Sperm motility (%)	87.5 ± 5.2	80–95	80.0 ± 9.1	70–90	84.5 ± 7.6
Progressive status (0-5)	3.9 ± 0.4	3.5–4.5	3.1 ± 0.5	2.5–3.5	3.6 ± 0.6
Total concentration ($x10^9$)	5.0 ± 2.6	2–10.4	6.1 ± 4.2	0.8 – 9.5	5.7 ± 3.4
Sperm viability (%)	84.7 ± 3.9	78–89	81.4 ± 10.7	69–92	83.4 ± 7.1
Intact acrosomes (%)	70.8 ± 8.3	59–81	72.0 ± 5.4	67–78	71.3 ± 6.9
Normal morphology (%)	81.5 ± 6.0	70–87	74.8 ± 21.4	47–93	78.8 ± 13.6
Seminal pH	8.0 ± 0.2	7.7–8.3	7.9 ± 1.0	7–9	8.0 ± 0.6
Collection Parameters	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD
Voltage	n/a	2–9	n/a	2–8	n/a
mAmps	n/a	50–460	n/a	70–360	n/a
# of stimuli	76.5 ± 25.5	55–113	87.7 ± 21.1	65–125	83.2 ± 22.3

was not significant ($P > 0.05$), therefore, data were combined for analysis. Data is presented as mean ± SD and statistical significance was defined as $P \leq 0.05$.

3. Results

Fresh ejaculate characteristics and collection parameters of black and Indian rhinoceros collected via electroejaculation are summarized in Table 2. CW treatment groups experienced an immediate and complete loss of motility and viability following initial dilution, therefore treatment was discontinued after two animals. One straw of 1%SL did not seal properly and was not included in statistical analysis.

Post-thaw motility (Fig. 1) and progressive status decreased ($P \leq 0.05$) in all groups when compared to fresh ejaculate, and were lower in all treatment groups than control ($P \leq 0.05$; Table 3). Motility and progressive status did not differ between 1%SL and 2% SL ($P > 0.05$), but were lower in CM and CW treatment groups than the SL treatment groups ($P \leq 0.05$), and decreased over time for EY, 1%SL, and 2%SL ($P \leq 0.05$; Fig. 1). Acrosomal integrity was similar ($P > 0.05$) in EY, 1%SL, and 2%SL, but lower ($P \leq 0.05$) in CW and CM (Table 3), and did not change over time in any treatment ($P > 0.05$; data not shown). Viability was greatest in EY and 2%SL followed by 1%SL, then CM and CW ($P \leq 0.05$; Table 3) and did not change over time in any of the treatment groups ($P > 0.05$; data not shown). Morphology did not differ among treatments (Table 3) or over time ($P > 0.05$) and most of the observed morphological abnormalities were secondary defects, specifically proximal and distal droplets (data not shown).

4. Discussion

To our knowledge, this is the first study to evaluate the use of soy lecithin, coconut water, and coconut milk as animal protein-free alternatives to traditional egg-yolk in rhinoceros semen extenders and perhaps the most comprehensive assessment of cryopreservation of black rhinoceros semen thus far. CW and CM were discounted as substitutes for EY due to complete loss of sperm viability in pre-freeze and post-thaw samples, respectively. The two SL based extenders (1 and 2%) initially demonstrated potential as possible EY replacements, with sperm exhibiting similar viability, morphology, and acrosomal integrity as those in the control. Unfortunately, there was a significant loss of sperm motility and progression in these treatments post-thaw. Therefore, SL appears to be an inferior alternative to EY in EQ-based media for cryopreserving rhinoceros sperm. However, these data provide a foundation upon which future studies can build in developing and optimizing an animal protein-free semen extender for rhinoceros.

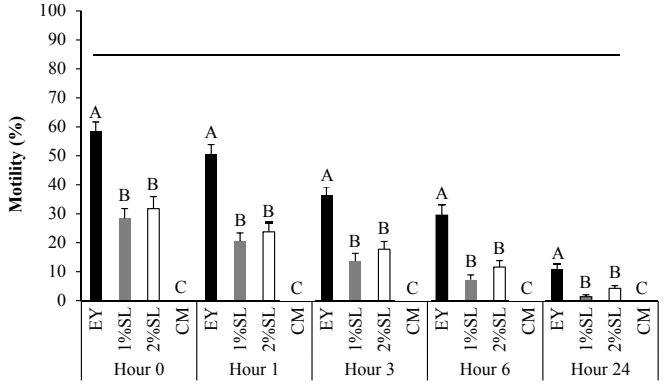

Fig. 1. Impact of semen extender treatment (egg-yolk (EY), 1% and 2% soy lecithin (1% SL; 2%SL), coconut milk (CM)), on black and Indian rhinoceros spermatozoa % motility post-thaw at 0, 1, 3, 6, and 24 h. Solid horizontal line indicates mean % motility in fresh samples. Data represent means ± SD. Different letters indicate significant differences between treatments within the indicated time point (n = 10).

Table 3
Post-thaw (hour 0) black and Indian rhinoceros sperm quality measures of each tested semen extender treatment (n = 10 for EY, 1%SL, 2%SL, and CM; n = 2 for CW).

	Motility (% motile)	Progression (0-5 scale)	Acrosomes (% intact)	Viability (% alive)	Morphology (% normal)
EY	58.5 ± 14.2^{A}	2.7 ± 0.7^{A}	52.3 ± 16.2^{A}	70.7 ± 10.3^{A}	78.3 ± 11.6^{A}
1%SL	28.4 ± 14.6^{B}	1.8 ± 1.6^{B}	50.0 ± 17.6^{A}	51.8 ± 13.2^{B}	82.3 ± 9.8^{A}
2%SL	31.8 ± 18.6^{B}	1.9 ± 0.8^{B}	57.1 ± 23.9^{A}	66.4 ± 12.2^{A}	81.1 ± 8.9^{A}
CM	0.0 ± 0.0^{C}	0.0 ± 0.0^{C}	8.7 ± 15.5^{B}	1.4 ± 3.7^{C}	87.0 ± 10.2^{A}
CW	0.0 ± 0.0^{C}	0.0 ± 0.0^{C}	2.0 ± 2.7^{B}	0.1 ± 0.3^{C}	93.5 ± 3.4^{A}

EY = egg-yolk, SL = soy lecithin, CM = coconut milk, CW = coconut water. Data presented as means ± SD. Different letters indicate statistically significant differences with each measure ($P \leq 0.05$).

Ejaculate characteristics of fractions selected for cryopreservation, both fresh and post-thaw, were similar to those previously reported for black, white, and Indian rhinoceros. Motility (84.5 ± 7.6%) and progressive status (3.6 ± 0.6) were comparable to earlier reports (~79–84%: 2.9 to 4.4, respectively) [8,10]. Semen collected in this study contained greater numbers of morphologically normal sperm (78.8 ± 13.6%) than that previously reported for samples cryopreserved from black and Indian rhinoceros (~42–56%) [8,10]. However, values were similar to those reported for sperm collected via electroejaculation from white rhinoceros (~73%) [47] and during gamete rescue from black and white rhinoceros (~70–90%) [4]. Fresh ejaculates with less than 60% sperm motility were excluded potentially inflating quality measures when compared to previous studies that may not have imposed the same lower quality limit. Ejaculate volume and total sperm concentration varied greatly among males (ranging 2.8–139.0 mL and 0.8 to 10.4×10^{9}, respectively). This range in values is probably not reflecting differences in fertility. Instead, it is likely due to inconsistency in individual responses to the same collection procedure, as previously suggested [8]. As many of the electroejaculation procedures were conducted opportunistically, often secondary to medical procedures, time of semen collection relative to anesthesia varied as did the anesthetic regimen employed. Both variables may influence semen output.

Percent motility and progressive status, measures of sperm quality, declined in all treatment groups including the control immediately post-thaw compared to pre-freeze samples. This result was expected as loss of motility following cryopreservation is common, though severity varies by species [8,10,48]. Cryosurvival is influenced by many factors including osmotic and cold tolerance, membrane composition and permeability of the spermatozoa, as well as use of cryoprotectants, extenders, and freezing/thawing rates [14,48]. Traditional extenders typically contain an animal protein, such as EY, which aids in protecting the cell membrane. Similar to results reported for bulls [18], goats [28], and rams [29], rhinoceros spermatozoa cryopreserved in extender with SL demonstrated viability (2%SL only), acrosomal integrity, and morphology values equal to the EY control. SL is speculated to protect cell membranes by preventing the loss of phospholipids through retention or replacement via its main component: phosphatidylcholine [11,12,25]. However, cell membrane stability is only part of the equation. Sperm must also retain their motility and function post-thaw.

Unfortunately, motility and progression were greatly hindered in the SL treatment groups. Upon microscopic examination, the post-thaw suspension appeared to contain viscous aggregates

which stuck to the spermatozoa and reduced their ability to move; flagella continued to beat but the spermatozoa appeared unable to pull away from the aggregates. This phenomenon occurred with both concentrations of SL and the impact did not appear to vary in severity between the two concentrations or among rhinoceros. In this study, 2%SL performed better than the 1%SL resulting in greater viability values, though motility was the same. This differs from reports in rams [29] and goats [28], which indicated a toxic effect of greater SL concentrations, that was attributed to increased viscosity as SL concentration increased. In a previous report by van Wagtendonk-de Leeuw et al., loss of bull sperm motility following cryopreservation in a soy-based extender was reported and speculated to be caused by the presence of debris and a higher viscosity [31]. However, this is not a universal response, as conflicting reports demonstrate less agglutination in samples cryopreserved in SL compared to EY [18,49] or mention no change in viscosity [21]. Such conflicting results may be due in part to extender quality or composition differences [50]; it may be useful to explore alternative extender compositions to rule out possible interactions of extender components with SL. However, the aggregates were not visible prior to freezing with semen, and therefore, it appears a component of the SL interacted with the seminal plasma as opposed to other extender components. Centrifugation and resuspension in new extender helped to remove some of the aggregates in this study, but resulted in further motility loss due to the mechanical stress as indicated by separation of sperm heads from flagella. Without an efficient method to avoid formation of the aggregates, SL is not an adequate replacement for EY in the media tested herein.

Immediately following the addition of the CW extender to semen of two individuals, spermatozoa were rendered immotile and nonviable. Acrosomal integrity plummeted, indicating probable membrane damage or induction of the acrosome reaction. Osmolality of the CW extender (~420 mmol/kg) was greater than that of the other extenders (~250–290 mmol/kg) and rhinoceros semen (~300 mmol/kg) [6,8,10,47]. Despite containing minimal cytoplasm, spermatozoa can dramatically respond to osmotic changes [14,51]. The CW extender created a hypertonic environment, potentially causing the efflux of water from the cell resulting in membrane damage, loss of acrosome, and cell death [14]. Because many acrosomes were observed separate from the sperm heads, it appears the decline is likely due to membrane damage. However, it is also possible CW is toxic to rhinoceros sperm, contains a component that interacts with the seminal plasma to create a toxic environment, or is capable of inducing an acrosome reaction. Immediate loss of viability was unexpected as CW has successfully been used as a semen extender for a variety of species including stallions [52], goats [37], and rams [36]. CW extenders are sometimes supplemented with EY, which may protect the membrane from any detrimental effects; however, post-thaw viability and membrane integrity of goat sperm were enhanced when cryopreserved in CW extender without EY [37]. Sperm membrane composition varies among species, in part dictates cold tolerance of sperm, and may also influence the cell's response to extenders [12,48]. The loss of viability of rhinoceros sperm due to CW addition may be species or taxonomic specific.

The response of rhinoceros sperm to CM extender was less pronounced than that to CW, but resulted in similar outcomes. Prior to freezing, spermatozoa extended with CM were viable and motile at values equal to EY and SL treatments. However, post-thaw sperm were nonviable. The osmolality of the CM extender was lower than that of CW and similar to that of EY and SL and did not appear to damage the membrane. CM was successfully used as an additive to extenders for bull and goat semen at room temperature and chilled [39,40]. However, there have not been any reports of a CM extender used in cryopreservation that did not also contain EY. Additionally, CM has greater amounts of PUFAs than coconut water [33,53] and as PUFAs promote membrane integrity and flexibility and sperm viability [16], CM was predicted to promote cryosurvival. As evidenced by the severe decline in viability and acrosomal integrity post-thaw, the fatty acids found in CM did not offer protection on par with EY and SL and is not recommended as a replacement for EY in semen extenders for black or Indian rhinoceros.

Many semen cryopreservation protocols, including those previously developed for rhinoceros species, rely on the use of an extender containing an animal-based protein, typically EY, to protect spermatozoa throughout freezing and thawing [8,10,12,31,44,48]. Our results demonstrate that rhinoceros sperm can survive cryopreservation without a source of animal-protein added to the cryoextender. SL appears to offer protection to rhinoceros sperm membranes during cryopreservation and, with some modification, may serve as an effective replacement for EY in traditional rhinoceros semen extenders. However, a method for eliminating aggregate formation post-thaw must be devised so that sperm motility is unencumbered. Alternatively, there may be other animal protein-free media that warrant testing. Ultimately, the development of an animal protein-free extender would ensure a more consistent composition and reduce the risk of avian disease transmission in cryopreserved rhinoceros semen.

Conflicts of interest

None.

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